Relation between retinoblastoma and p53 proteins in human papilloma viruses 16/18 positive and negative cancers of the uterine cervix

R Chetty, A Bramdev, A Aguirre-Arteta, R J Pegoraro, N Sataar

Abstract

***Aim*—To ascertain the extent of retinoblastoma protein (pRB) expression in comparison to p53 protein and human papilloma viruses (HPV) 16/18 status in cervical carcinomas.**

***Methods*—Fifty cases of invasive cervical carcinoma were HPV typed for genotypes 16 and 18 using consensus primers by polymerase chain reaction (PCR). Immunohistochemistry for pRB and p53 was done on formalin fixed tissue using microwave antigen retrieval and commercially available antibodies.**

***Results*—Forty five cases were squamous carcinomas, three were adenocarcinomas, and two were adenosquamous carcinomas. Thirty one cases were HPV 16 positive and one was HPV 18. Sixteen cases showed +4 pRB expression and a further 11 were +3 positive. Seven cases were negative. Only five cases (10%) showed +4 p53 immunostaining, while seven were negative and 15 were +1. Of the 16 pRB +4 positive cases, one was negative for p53 and a further seven were +1 positive. This inverse pattern of staining between pRB and p53 had a p value of <0.001. No correlation was observed between HPV 16/18 status and p53 and/or pRB staining.**

***Conclusions*—pRB is expressed in the majority of cases of cervical cancer (86%), with more than 75% (+4) of the tumour cell population being positive in 16 cases (32%). There appears to be a general inverse pattern of staining between pRB (high) and p53 (low) in cervical cancer. The expression of both pRB and p53 proteins is independent of the HPV 16/18 status of the tumour.**

(*J Clin Pathol* 1997;50:413–416)

Keywords: retinoblastoma; p53; human papilloma virus; uterine cervical carcinoma

University of Natal School of Medicine, Durban, South Africa: Department of Anatomical Pathology
R Chetty
A Bramdev
N Sataar

Department of Chemical Pathology
A Aguirre-Arteta
R J Pegoraro

Correspondence to:
Professor Runjan Chetty, Department of Anatomical Pathology, University of Natal School of Medicine, Private Bag 7, Congella 4013, Durban, Natal, South Africa.

Accepted for publication 22 January 1997

There is a strong association between particular genotypes of human papilloma virus (HPV) and cervical cancer. The mechanism by which HPV achieves oncogenesis is thought to be mediated by two viral oncoproteins, E6 and E7.[1] Both these oncoproteins complex with several cellular proteins that are responsible for regulating cell growth, especially p53 and retinoblastoma (pRB) proteins. Both these proteins are products of tumour suppressor genes that normally maintain tightly regulated cell proliferation and growth. When the control mechanisms of the cell cycle are disturbed, unrestricted cell proliferation and ultimately the tumour state occurs.

The purpose of this paper is to explore the extent of pRB and p53 immunoexpression and to see if a relation exists between these two proteins, in the setting of both HPV 16/18 positive and negative cervical cancers from South Africa.

Methods

Fifty consecutive cases diagnosed as carcinoma of the uterine cervix were used in this study. Fresh tissue was collected for DNA extraction and surgical biopsies were also submitted in 10% buffered formalin. All 50 cases were evaluated by two of us (RC and AB) to confirm the diagnosis, type, and grade of carcinoma.

DNA extraction was accomplished from fresh tissue using a modification of the salting out method of Miller *et al.*[2] Tumour DNA was analysed for the presence of HPV by polymerase chain reaction (PCR) amplification using the L1 consensus primers MY 09 and MY 11, and the cycling conditions recommended by Manos *et al.*[3] Amplified HPV DNA was typed for HPV 16 and HPV 18 by Southern hybridisation using specific oligonucleotide probes labelled with digoxygenin (Boehringer Mannheim, Germany). Cervical cancers known to be HPV 16 and 18 positive were used as positive controls. Reactive tonsil was used as negative control tissue. Amplifiable DNA was verified by amplification of the β globin gene (approximately 300 base pairs in size).

Immunohistochemistry was performed on formalin fixed, paraffin embedded tissue using the streptavidin-biotin complex technique with DAB as chromogen. Antigen retrieval was performed with microwave pretreatment. Antibodies used were: DO7 to detect p53 protein (monoclonal, dilution 1 in 1000, Dakopatts, Denmark) and pRB to detect pRB (monoclonal, dilution 1 in 50, Zymed Laboratories, USA). DO7 detects both wild-type and mutant p53 protein, while the antibody to pRB detects both the phosphorylated and unphosphorylated form of the protein. Appropriate positive controls (a case of colorectal cancer which was known to be immunopositive with both p53 and pRB) and negative control (omission of the primary antibody) were employed.

The quantitation of immunostaining for p53 and pRB was assessed as follows: less than 5%

Table 1 Summary of results

Case	Histologica	Grade	HPV	p53	pRb
1	SCC	Poor	16	2+	–
2	SCC	Mod	16	2+	–
3	AC	Poor	–	–	2+
4	SCC	Poor	16	1+	1+
5	SCC	Mod	16	1+	1+
6	SCC	Mod	16	3+	–
7	SCC	Mod	16	1+	4+
8	SCC	Mod	16	2+	4+
9	SCC	Mod	–	–	1+
10	SCC	Mod	16	–	2+
11	SCC	Mod	–	2+	2+
12	SCC	Mod	16	–	4+
13	SCC	Poor	16	3+	–
14	SCC	Well	–	1+	1+
15	SCC	Well	–	3+	3+
16	SCC	Mod	16	1+	3+
17	SCC	Mod	16	2+	3+
18	SCC	Poor	16	1+	3+
19	SCC	Mod	16	3+	3+
20	SCC	Mod	16	3+	3+
21	SCC	Mod	16	4+	2+
22	SCC	Mod	–	2+	4+
23	SCC	Mod	16	2+	3+
24	SCC	Mod	16	1+	1+
25	SCC	Poor	18	2+	4+
26	SCC	Mod	16	1+	4+
27	AC	Poor	–	–	–
28	ASC	–	–	1+	4+
29	ASC	–	–	1+	4+
30	SCC	Mod	16	1+	3+
31	SCC	Poor	–	2+	4+
32	SCC	Poor	16	2+	4+
33	SCC	Mod	–	3+	4+
34	SCC	Poor	16	–	–
35	SCC	Mod	16	3+	2+
36	SCC	Poor	–	2+	2+
37	SCC	Mod	–	4+	4+
38	SCC	Mod	–	2+	4+
39	SCC	Mod	16	4+	2+
40	SCC	Poor	16	–	1+
41	SCC	Mod	–	2+	1+
42	SCC	Mod	–	1+	–
43	AC	Mod	16	4+	3+
44	SCC	Mod	16	2+	1+
45	SCC	Mod	16	3+	3+
46	SCC	Well	16	4+	2+
47	SCC	Mod	16	1+	4+
48	SCC	Poor	–	2+	3+
49	SCC	Well	–	1+	4+
50	SCC	Poor	16	1+	4+

AC, adenocarcinoma; ASC, adenosquamous carcinoma; SCC, squamous cell carcinoma.

of cells immunolabelled, negative; 5–24% cells, positive +1; 25–49% +2; 50–74% +3; 75–100% +4.

STATISTICS

A Fisher's exact test and a χ^2 test were applied for statistical analysis, and a p value < 0.05 was considered significant.

Results

The results are summarised in table 1. Forty five of the cases were squamous carcinomas, three were adenocarcinomas, and two were adenosquamous carcinomas. The degree of differentiation of the squamous carcinomas was as follows: four were well differentiated, 29 were moderately differentiated, and 12 were poorly differentiated. Of the three adenocarcinomas, two were poorly differentiated, the other being moderately differentiated. In total 32 (31 squamous carcinomas and one adenocarcinoma) of the 50 cases were either HPV 16 or 18 genotypes (fig 1). Thirty squamous carcinomas and the one moderately differentiated adenocarcinoma were HPV 16 positive, while only one squamous carcinoma (poorly differentiated) harboured HPV 18.

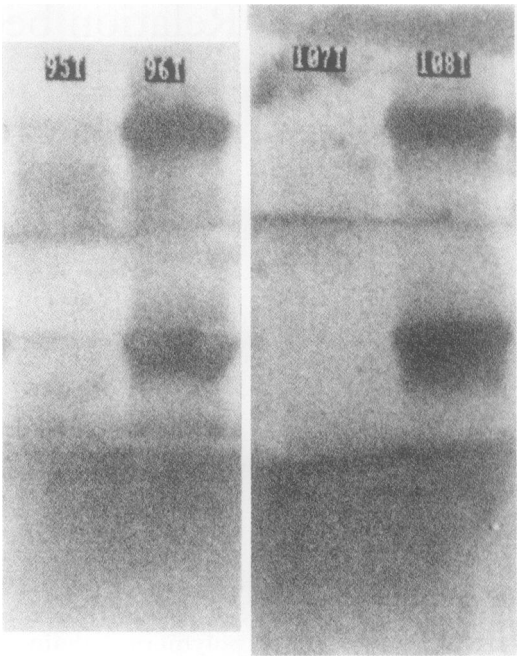

Figure 1 Nylon blots showing hybridisation of generic HPV (upper bands) and HPV 16 (lower bands) in two positive cases (96 T and 108 T).

pRB EXPRESSION

Sixteen cases showed +4 staining (fig 2) and a further 11 were +3 positive. Seven cases were negative, eight showed +1 staining, and a further eight were +2 positive.

p53 IMMUNOSTAINING

Only five cases showed +4 positivity (fig 3), while seven were negative and 15 showed +1 and +2 immunoreactivity. Eight cases showed +3 positivity.

Of the 16 cases that were +4 pRB positive, the corresponding p53 immunostaining was as follows: one was negative, seven showed +1, six were +2, and one case each was +3 and +4 ($p < 0.001$). Of the five cases that were +4 for p53, three were +2 for pRB. One of the adenocarcinomas (case 43) showed +4 p53 staining (fig 4).

No statistical correlation was observed between HPV 16/18 status and p53 or pRB immunostaining.

Discussion

The role of tumour suppressor genes in the process of carcinogenesis is an important one and is currently being investigated intensively. Both p53 and Rb are prototype tumour suppressor genes and defects within these two genes result in loss of their oncosuppressor functions. Mutant p53 protein probably acts as a dominant suppressor of the wild-type protein. Wild-type p53 protein has the ability of arresting cells in G1 in response to any genotoxic event and inducing programmed cell death if the DNA damage cannot be repaired, or if aberrent replication cannot be stopped.[4]

The retinoblastoma gene, Rb, is deleted or mutated in several malignancies including retinoblastomas, osteosarcomas, lung, breast, and bladder cancers.[5–9] Loss of the Rb gene is thought to lead to loss of pRB protein with

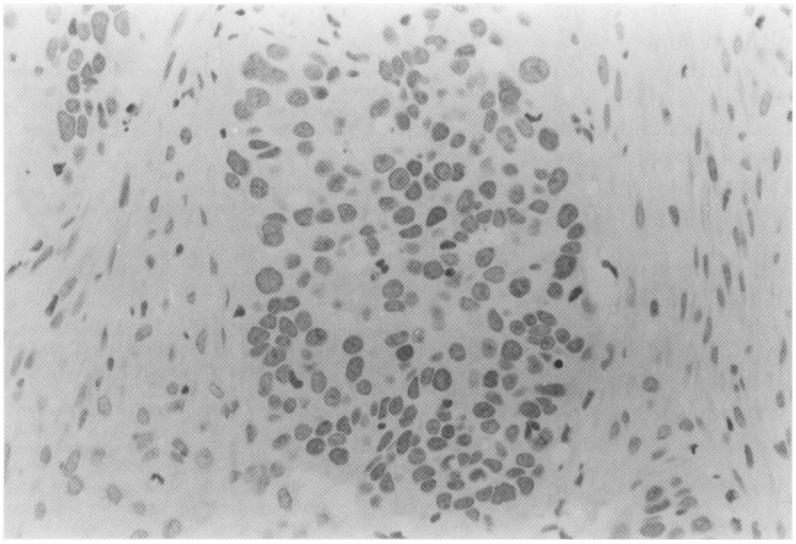

Figure 2 A moderately differentiated squamous carcinoma showing +4 staining for pRB. Staining appeared slightly heterogeneous, with some negative nuclei present (anti-pRB, ×87).

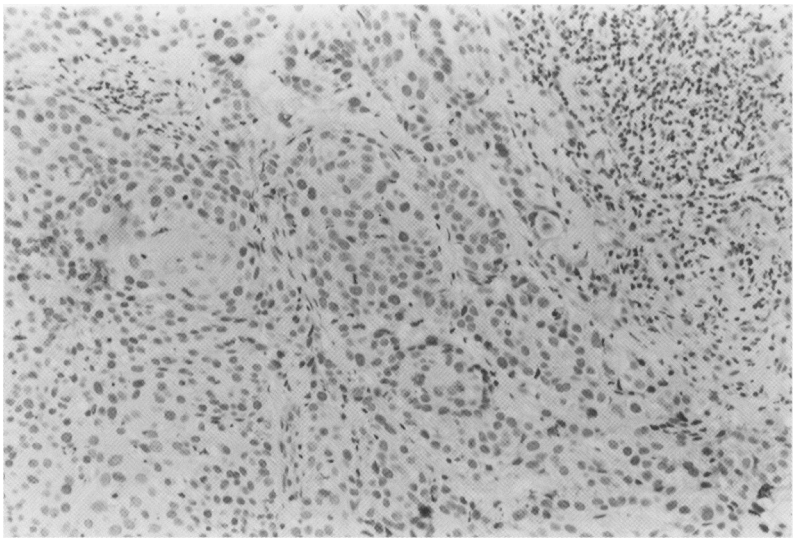

Figure 3 p53 immunoexpression in an infiltrating squamous carcinoma. Almost all the tumour cell nuclei are stained and this was regarded as +4 positivity. Only five cases showed this intensity of staining (anti-p53, ×22).

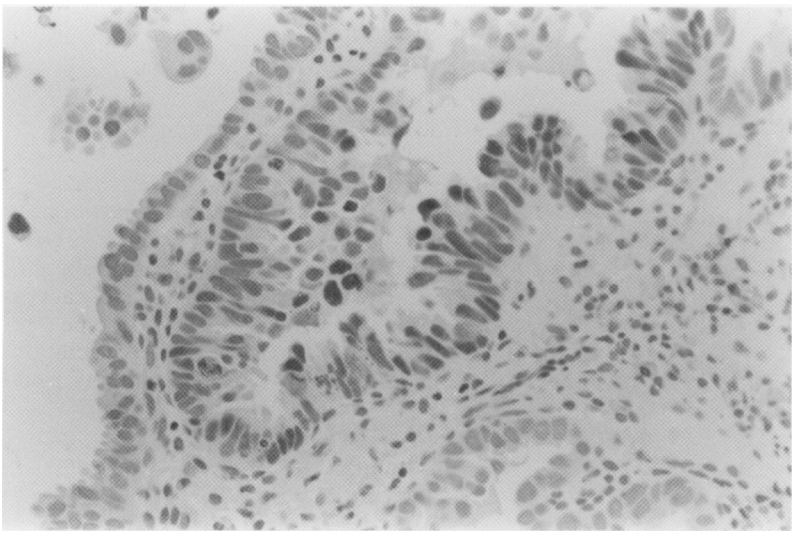

Figure 4 The moderately differentiated adenocarcinoma which was HPV 16 positive, showing +4 p53 expression. Note the in situ adenocarcinoma component also showing intense immunolabelling for p53 (anti-p53, ×87).

consequent uncontrolled cell proliferation, and eventually tumour formation.

Central to uterine cervical carcinogenesis is the role of the high risk HPV genotypes: 16, 18, and 31.[10 11] The precise mechanism by which HPV induces cervical carcinogenesis has proved somewhat enigmatic. However, it has emerged that simple HPV infection alone is not sufficient for oncogenesis. Rather, integration of viral DNA into host DNA is the key.[12-15] On integration of the HPV genome, there is disruption of the E2 open reading frame while the E6 and E7 regions remain intact.[1] It is thought that these two viral genes and their proteins are responsible for the transforming ability of HPV.[16]

E7 binds to pRB and it would be logical to suggest that this E7–pRB complex would lead to loss of negative control of the cell cycle leading to uncontrolled cell proliferation. Similarly, E6 interferes with the normal function of p53 by causing degradation of wild-type p53. This is enhanced by the fact that E6 has similar properties to mutant p53.[1]

The results of our study do not point to a straightforward relation between HPV 16/18 and pRB and p53. With regard to p53 protein expression, we have shown immunopositivity in both HPV 16/18 positive and negative cervical cancers, as have others.[17] To explain these findings, it has been postulated that there are conformational changes in wild-type p53 protein, causing it to change from suppressor to promoter or mutant p53. Others have found both p53 protein and gene abnormalities to be infrequent in cervical cancers.[18] Low levels of p53 protein expression were also found in the current study, irrespective of HPV 16/18 status.

The functional state of pRB is dependent on its state of phosphorylation, which occurs in a cell cycle dependent manner.[19] It is the under- or hypophosphorylated form of pRB that functions as a cell cycle regulator. It is also known that several adenoviruses and other DNA viruses bind to the hypophosphorylated form of pRB. The expression of pRB has not been intensively studied in human cervical cancers along with the HPV status. In a study on cervical cancer cell lines, it was suggested that E7 protein expression results in loss of pRB gene function in HPV positive cell lines.[20]

In 27 of 50 cervical carcinomas analysed in this study, more than 50% of the tumour cell population expressed pRB. Like p53 immunoexpression, pRB immunoreactivity was independent of HPV 16/18 status. Of the 32 HPV 16/18 positive tumours, 17 displayed +3 or +4 pRB immunolabelling of tumour cells. From these results it would appear that E7 inactivation of pRB does not occur in the majority of cases. There could be several reasons for this increased pRB expression. It may well be that E7–pRB complexes do form in cervical cancer, and immunohistochemistry is detecting only the hyperphosphorylated form of pRB. Alternatively, the complex may be formed and turned over rapidly, or the complex may occur early in HPV infection and be lost during the later stages of carcinogenesis.[21]

An interesting facet to this study lay in the comparison of p53 and pRB immunoprofiles. Those cases with high pRB expression tended to have low immunoexpression of p53. This inverse pattern of staining has also been seen in colorectal adenocarcinomas.[22] The significance of this is not readily apparent, but may be a reflection of low p53 expression in general in cervical cancers.

This study has therefore confirmed that p53 protein expression (which is independent of HPV 16/18 status) is low in cervical cancers. pRB immunostaining on the other hand is high, and this too is independent of HPV 16/18 infection. Of interest is the inverse relation between pRB (high) and p53 (low) proteins in cervical cancer.

1 Vousden K. Interactions of human papillomavirus transforming proteins with the products of tumor suppressor genes. *FASEB J* 1993;**7:**872–9.
2 Miller Sa, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acid Res* 1988;**16:**1215.
3 Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM. The use of polymerase chain reaction for the detection of human papillomaviruses. In: Firth M, Greaves M, eds. *Molecular diagnostics of human cancer: cancer cells.* New York: Cold Spring Harbour, 209–14.
4 Moran E. Interaction of adenoviral proteins with pRB and p53. *FASEB J* 1993;**7:**880–5.
5 Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, *et al.* A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 1986;**323:**643–6.
6 Hansen MF, Koufos A, Gallie BL, Phillips RA, Fodstad O, Brogger A, *et al.* Osteosarcoma and retinoblastoma: a shared chromosomal mechanism revealing recessive predisposition. *Proc Natl Acad Sci (USA)* 1985;**82:**6216–20.
7 T'Ang A, Varley JM, Chakraborty S, Murphree AL, Fung YK. Structural rearrangement of the retinoblastoma gene in human breast carcinoma. *Science* 1988;**242:**263–6.
8 Yokota J, Akijama T, Fung YK, Benedict WF, Namba Y, Hanaoka M, *et al.* Altered expression of retinoblastoma (RB) gene in small cell carcinoma of the lung. *Oncogene* 1988;**3:**471–5.
9 Logothetis CJ, Xu H-J, Ro JY, Hu S-X, Sahin A, Ordonez G, *et al.* Altered expression of retinoblastoma protein and known prognostic variables in locally advanced bladder cancer. *J Natl Cancer Inst* 1992;**84:**1256–61.
10 Schiffman MH. Recent progress in defining the epidemiology of human papillomavirus infection and cervical neoplasia. *J Natl Cancer Inst* 1992;**84:**394–7.
11 Herrington CS. Human papillomaviruses and cervical neoplasia. I. Classification, virology, pathology, and epidemiology. *J Clin Pathol* 1994;**47:**1066–72.
12 Cooper K, Herrington CS, Stickland JE, Evans MF, McGee JO'D. Episomal and integrated HPV in cervical neoplasia demonstrated by non-isotopic in situ hybridisation. *J Clin Pathol* 1991;**44:**990–6.
13 Cullen AP, Reid R, Campion M, Loriez AT. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasms. *J Virol* 1991;**65:**606–12.
14 Das BC, Sharma JK, Gopalakrishna V, Luthra UK. Analysis by polymerase chain reaction of the physical state of human papillomavirus type 16 in cervical preneoplastic and neoplastic lesions. *J Gen Virol* 1992;**73:**2327–36.
15 Lehn H, Villa LL, Marziona F, Hilgarth M, Hillemans HG, Sauer G. Physical state and biological activity of human papillomavirus genomes in precancerous lesions of the female genital tract. *J Gen Virol* 1988;**69:**187–96.
16 Mansur CP, Androphy EJ. Cellular transformation by papillomavirus oncoproteins. *Biochim Biophys Acta* 1993;**1155:** 323–45.
17 Cooper K, Herrington CS, Evans MF, Gatter KC, McGee JO'D. p53 antigen in cervical condylomata, intraepithelial neoplasia, and carcinoma: relationship to HPV infection and integration. *J Pathol* 1993;**171:**27–34.
18 Busby-Earle RMC, Steel CM, Williams ARW, Cohen B, Bird CC. p53 mutations in cervical carcinogenesis—low frequency and lack of correlation with human papillomavirus status. *Br J Cancer* 1994;**69:**732–7.
19 Mittnacht S, Weinberg RA. G1/S phosphorylation of the retinoblastoma protein is associated with an altered affinity for the nuclear compartment. *Cell* 1991;**65:**381–93.
20 Scheffner M, Münger K, Byrne JC, Howley PM. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci USA* 1991;**88:**5523–7.
21 Dyson N, Howley PM, Münger K, Harlow E. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989;**243:**934–6.
22 Chetty R, Subramoney T, Singh JP, Harilal P. Retinoblastoma (pRb) and p53 protein immunoexpression in sporadic colorectal cancer. *Anticancer Res.* [In press.]